Functional Innervation of Cultured Skin Grafts

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The aims of the present study were to determine 1) if grafts of cultured skin become innervated; and 2) whether tactile function of these grafts could be improved by implanting target tissue into them.

Autologous skin equivalents were generated in vitro (30 d) for individual adult Sprague-Dawley rats. Some animals received pure skin equivalent grafts; others had target tissue consisting of 2-mm punch biopsies (normal skin or touch domes) inserted into their skin equivalents at the time of grafting. After 83 d, physiologic recordings were obtained from afferent nerves innervating the grafts. Tissue was processed for histology at various intervals.

Silver staining of the tissues demonstrated many isolated nerve fibers in the dermis of cultured areas of skin as well as in implant zones. When grafts were rubbed with a glass rod or pinched with watchmaker forceps, impulses were evoked in nerves innervating both implant and cultured regions. In contrast, the afferent response to gently stroking grafts with a camel hair brush was severely reduced in cultured areas but was vigorous in implanted skin. Neuronal activity characteristic of type I neurons innervating touch domes was only found in cutaneous nerves innervating implants originally possessing domal tissue. Furthermore, grafts with good takes had better return of sensory function than grafts undergoing episodes of crusting.

These results suggest that structural components or trophic factors present in implants enhanced the return of neural function related to the sensory modality of light touch; and this was also affected by the engraftment quality. *J Invest Dermatol 99:120–128, 1992*

Between 1974 and 1986, the survival rate of individuals incurring massive burn injuries increased dramatically. This was largely due to improved techniques such as prompt excision of burn eschar and immediate wound closure either with skin autografts or cadaver allografts, followed by later treatment with autografts of cultured skin [1].

The use of cultured epidermis to cover burns on more than 50% of the body surface was first reported in 1984 [2]. Within 5 years, approximately 200 patients had been treated [3]. Skin provides a protective barrier for the body, but in addition it conveys information about the environment via specialized sensory receptors. However, cultured skin has been shown to lack adnexal structures for as long as 5 years post-grafting [4], and therefore probably lacks the highly organized nerve endings around hair follicles. Following recovery from burn-grafting procedures, individuals must be concerned about the return of their cutaneous sensation. As the use of cultured grafts increases, it will become important to improve methodologies so that graft quality is maximized for individual recipi-

Within skin are specialized areas called touch domes that are richly innervated. These structures can be seen on the skin surface of mammals following depilation. Histologically, they are characterized by an elevated dermal papillae covered with a thickened epidermis that has Merkel cells in the basal epidermal layer. Merkel cells possess dense-cored granules and are innervated by afferent type I mechanoreceptive neurons [5] having a characteristic slowly adapting irregular pattern of discharge that can be identified with a hand-held stimulus [6]. Touch domes are known to provide tropic or trophic factors for regenerating type I nerves [7].

The experimental design in the present investigation was to compare tactile sensory recovery in grafts composed entirely of cultured skin to that in cultured skin containing implants of normal skin with, or without, touch domes. The results indicate that target tissue (both normal hairy skin and touch dome hairy skin) improves sensory recovery. To our knowledge, this is the first study using both histologic and physiologic measurements to investigate sensory innervation in grafts of cultured skin. Preliminary reports of this work have appeared elsewhere [8,9].

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Abbreviations:
A: area of an ellipse
a: radius of the major axis
b: radius of the minor axis
DMEM: Dublecco's modified Eagle's medium
KGM: keratinocyte growth medium
PBS: phosphate-buffered saline
SE: skin equivalent
SE+ND: skin equivalent containing punch biopsies of non-domal or normal hairy skin
SE+TD: skin equivalent containing punch biopsies with touch domes

MATERIALS AND METHODS

Skin Grafts Autologous skin equivalents were cultured for approximately 3 weeks and subsequently grafted to the adult Sprague-Dawley rats (n = 13) that originally provided cells and implants [9–13]. Briefly, small (5-mm^2) biopsies were taken from the skin of individual animals and fibroblasts allowed to grow out from the dermis for 10 d in 25-cm^2 tissue culture flasks. The fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM; Hazleton,

Biologics, Inc.) plus 10% fetal calf serum to near confluency and passaged using 0.05% trypsin (Difco, 1:250) in phosphate-buffered saline (PBS). Third-passaged fibroblasts (4×10^5 cells/ml) were mixed with 2 × DMEM and evenly distributed by swirling in a 150 × 25-mm bacteriologic grade dish after which a solution of rat-tail collagen (2 mg/ml) was added to the dish. The collagen gelled within minutes and was allowed to contract into a disk for 2–3 d. At this time, keratinocytes obtained from a 2-cm² biopsy taken from the same animal were dissociated and seeded on top of the disk at a density of $3-6 \times 10^4/cm^2$. The following two growth media were employed for keratinocyte culture: i) DMEM with 10% fetal bovine serum (Hyclone) plus supplements [10^{-10} M cholera toxin (Sigma), 10 ng/ml epidermal growth factor (Collaborative Research), 0.4 μg/ml hydrocortisone (Sigma) and Fungizone, Penicillin, Streptomycin (Irvine Sci.)]; or ii) keratinocyte growth medium (KGM; Clonetics).

Skin equivalents were held in culture until the keratinocytes covered the surface of the fibroblast contracted gel (about 2 weeks), and were subsequently air exposed for 5–10 d. They were then grafted as full-thickness grafts (40 mm in diameter) to previously prepared sites on each animal's back.

As shown in Table I, six animals received grafts composed entirely of skin-equivalent tissue (SE). Three others received skin equivalents containing punch biopsies of normal hairy skin without touch domes (SE+ND), and four received skin equivalents with punch biopsies containing touch domes (SE+TD). The dermis of each biopsy plug was trimmed of fat and the surface of the plug aligned level with the surface of the skin equivalent. Skin equivalents received three plugs that were spaced about 6 mm apart from each other (Fig 1). For control purposes, an additional nine animals received full-thickness autografts of their own skin, and four others received full-thickness open wounds similar in size to the cultured grafts. Sterile technique was used for all procedures, and animals were housed individually. With time, grafts became ellipsoid in shape; therefore, their surface area was estimated using the equation $A = \pi ab$ (a, radius of the major axis; b, radius of the minor axis).

Nerve Function In several animals (SE = 2; SE+ND = 2; SE+TD = 3) nerve fields were defined before and after grafting by direct recording. The dorsal cutaneous nerves at levels T5–T13 were freed of connective tissue either where they exited the vertebrae between the ribs or where they emerged from the back muscles. Each nerve trunk was carefully cleaned and placed across bipolar platinum electrodes immersed in an oil pool. Nerve fields were roughly outlined on the skin surface by stroking the skin with a camel hair brush and marking the border with ink and tattoos. After 83 d post-grafting, the impulses generated in the nerves following stimulation of their cutaneous fields (stroking with a camel hair brush, rubbing with a glass rod, or gently pinching with fine forceps) were monitored on an oscilloscope and an audio speaker system, and recorded on magnetic tape for later analysis. Nerves serving normal skin rostral and caudal to the grafts were also recorded during cutaneous stimulation for each animal. Some fascicles were small enough for activity of individual nerve fibers to be monitored during gross recording (Fig 10). Artifacts caused by heartbeat and respiration were minimized with a band-pass filter (Kron-Hite).

Table I. Experimental Treatment of 26 Adult Rats

Type of Graft or Treatment	Number of Days on Animal	Number of Animals
Skin equivalent (SE) (keratinocytes cultured on the surface of a fibroblast populated collagen gel)	14–43	3
	395–468 (>1 year)	3
Skin equivalent plus non-domal implants (SE+ND)	83–97	3
Skin equivalent plus touch dome implants (SE+TD)	83–86	3
	899 (>2 years)	1
Autografts	16–44	2
	60–180	5
	460–497 (>1 year)	2
Open wounds	16–44	4

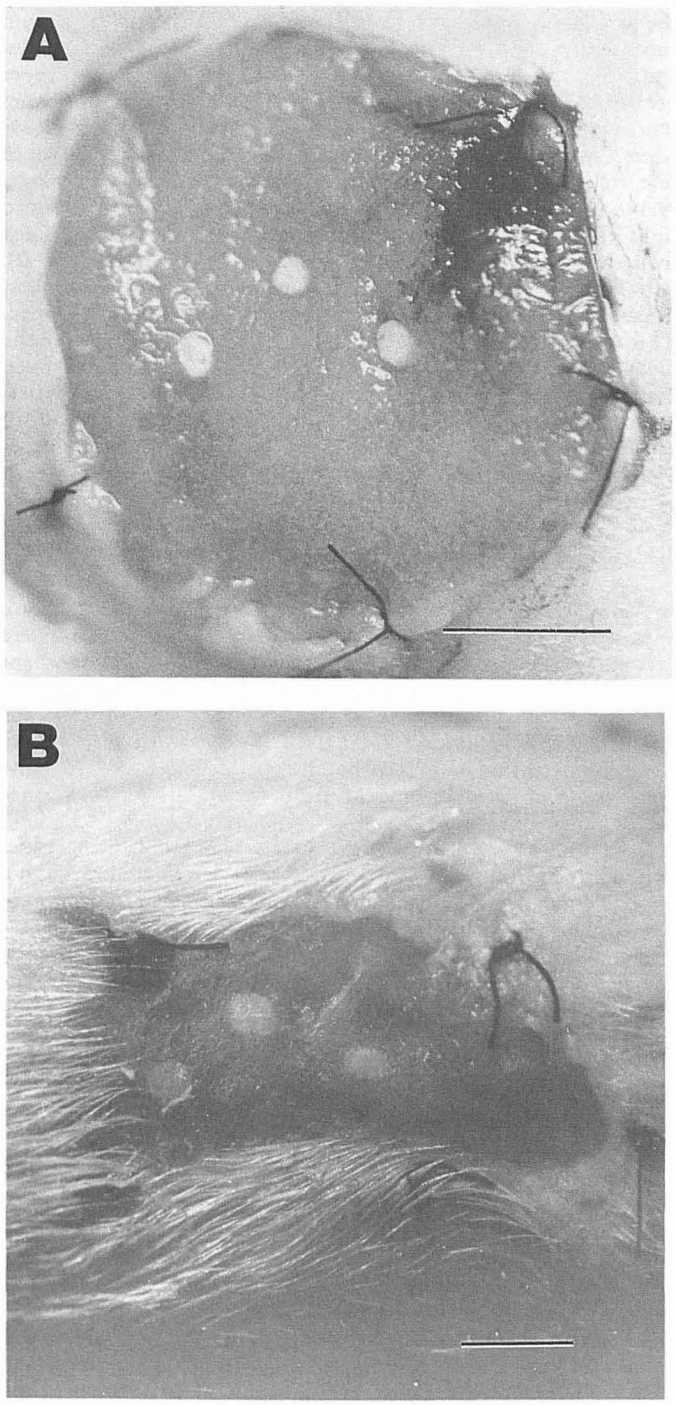

Figure 1. *A)* A skin equivalent at the time of grafting. Three 2-mm punch biopsies with touch-dome mechanoreceptors were trimmed of fat and inserted into the cultured skin. A little bleeding occurred at the suture placed about 2 o'clock. *Scale bar,* 10 mm. *B)* The same skin equivalent at bandage removal. The 2-mm punch biopsies are easily distinguished from the more translucent areas of cultured skin. *Scale bar,* 5 mm.

Nerve Counts and Measurements For nerve histology, tissue (5 mm²) was immersed in cold isopentane, frozen in liquid nitrogen, cryoprotected in OCT embedding medium for frozen tissue specimens (Miles, Inc.), sectioned 30 μm thick, and silver-stained following a modified Loots procedure [14,15]. Nerve-fiber profiles were counted in the dermis of skin equivalent grafts in regions between

the implants. Implant regions were easily distinguished from surrounding cultured skin, due to their organized collagen bundles and hair follicles. Counts were not made in the implants proper because the thick collagen bundles of the implant dermis sometimes obscured the silver-stained nerve fibers. In skin equivalent areas adnexal structures were missing, and the dermis was composed of fine collagen fibrils and numerous blood vessels and fibroblasts. Nerve fibers stained black and the dermis and epidermis stained light brown. Six sections were selected at random and examined from each animal (see Table I: SE = 2, SE+ND = 2, SE+TD = 2). After inspection of a section, the region judged to have the highest density of nerve-fiber profiles was positioned under an ocular grid. This was necessary because nerve fibers were distributed like the branches of a tree. Therefore, counts made in regions between branches would not have been representative of the degree of dermal innervation. For each square of the grid, a count was made of each nerve (magnification × 200) and its divisions as the microscope was focused throughout the 30-μm-thick section. For each section, an area three grids across and three grids down was mapped (1.44 mm^2). In addition, measurements were made of the nearest distance of nerves from the epidermal basement lamina.

In Vitro Controls to Determine if Skin Equivalent Tissue Was a Barrier for Nerve Growth Dorsal root ganglia were excised from newborn rat fetuses and placed into skin-equivalent collagen solution before it gelled. Keratinocytes were seeded on top of the contracted collagen disk 2 d later. Growth medium was supplemented with glucose (6 mg/ml), 10% heat-inactivated horse serum (Gibco), and 10% fetal bovine serum (KC Biol.). In some preparations, a 40:60 mixture of this medium with KGM (Clonetics) was also employed.

Co-cultures were fixed with phosphate-buffered 2% glutaraldehyde for 1 h, post-fixed in 2% osmic acid, dehydrated through a series of ethanol, and embedded in Araldite. Ultrathin sections were stained with uranyl acetate followed by lead citrate [16]. Alternatively, cultures were also fixed in 10% neutral-buffered formalin, embedded in paraffin, and Bodian-stained to reveal neurite outgrowth.

Graft Histology Tissue was processed by conventional methods for light and electron microscopy or fixed in 10% neutral-buffered formalin, paraffin embedded, and stained with hematoxylin and eosin.

RESULTS

Graft Histology

Skin Equivalents In Vivtro: Skin equivalents acquired a "fleshy" color as their keratinocytes became confluent and stratified upon air exposure; and when ready for grafting, they were firm and easy to handle. The epidermis covering the collagen gel was multilayered (Fig 2). Desmosomes joined keratinocytes to one another, and membrane-coating granules and sparse clumps of tonofibrils were present (Fig 3). Differentiated keratinocytes in the upper layers had keratohyalin granules. Rough endoplasmic reticuli, mitochondria, and Golgi apparati were numerous. Basal cells were often observed in mitosis. A basement membrane was lacking, but small hemidesmosome-like structures were present; and there were areas at the epidermal-dermal junction suggestive of enhanced fibril formation (Fig 4). In the first cultures, fibroblasts were sometimes mixed in with the epidermal keratinocytes, but as the methodology of splitting the epidermis from the dermis of 25-cm^2 biopsies improved the keratinocyte layers were essentially pure.

Skin Equivalents 35 d Post-Grafting: In general, the dermis of cultured areas outside of implant regions was reorganized and had fine fibrils of collagen and numerous blood vessels. The dermal collagen of the implants, however, retained much of its original structure. Both implant and skin equivalent areas had a hypertrophic epidermis.

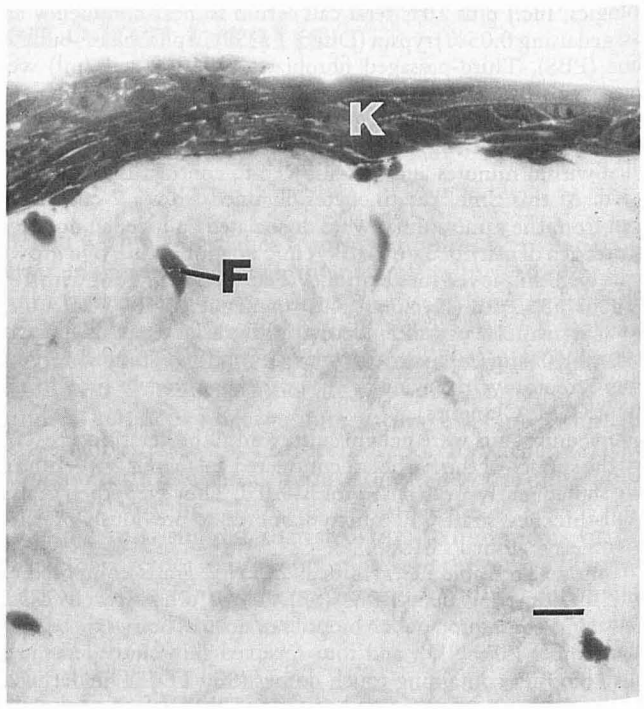

Figure 2. A methylene blue stained section of a skin equivalent 14 d in vitro. After 2–3 d in vitro, dissociated autologous keratinocytes were seeded on top of a collagen gel populated with fibroblasts (F). At 14 d, the keratinocytes were confluent and had stratified. *Scale bar,* 10 μm.

Figure 3. The epidermis of a skin equivalent 12 d in vitro after 2 d of air exposure. Tonofibrils (t), membrane coating granules (mcg), and desmosomes (d) were present. *Scale bar,* 0.50 μm.

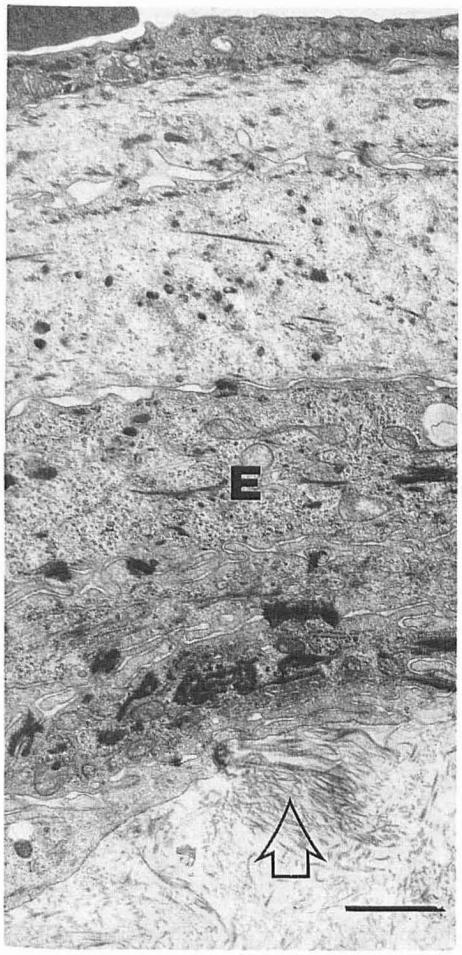

Figure 4. Skin equivalent 12 d in vitro. Areas of enhanced collagen formation *(arrow)* were present at the junction of the epidermis (E) and dermis. *Scale bar,* 1 μm.

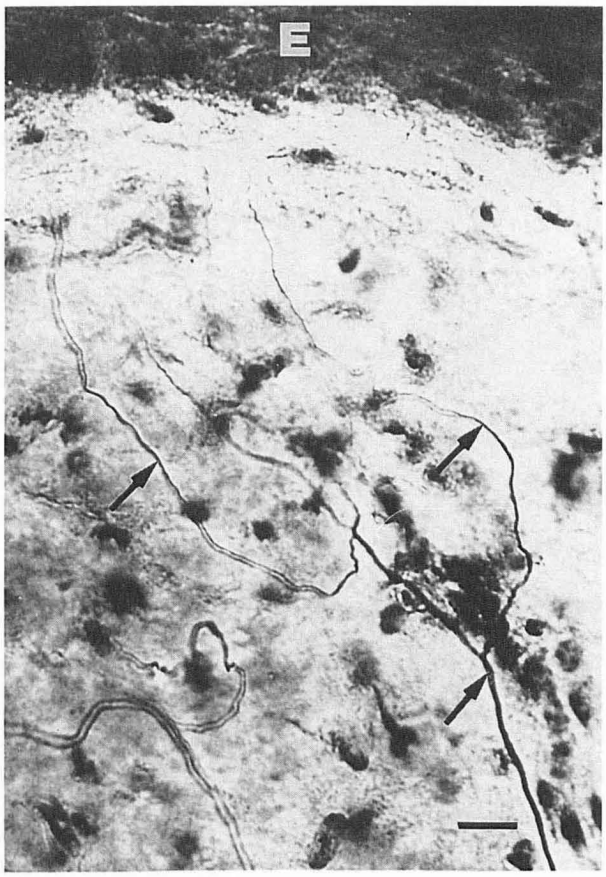

Figure 5. Cultured skin 83 d post-grafting. Silver-stained nerves *(arrows)* in the dermis of cultured skin outside of a touch dome implant. *Scale bar,* 10 μm.

Skin Equivalents After 83 d Post-Grafting: Nerves were present in skin equivalent and implant regions. Nerve fibers in the dermis of cultured skin were mostly isolated and followed blood vessels or were distributed in a seemingly random fashion. Individual axons wove a tortuous course in the dermis and often extended to the epidermis (Fig 5). In contrast, nerves going to the implants were often in bundles (Fig 6).

Skin Equivalents 1 Year Post-Grafting: The epidermis initially generated in vitro remained thicker than that of adjacent normal skin, and the dermis maintained its reorganized structure. Adnexae were missing (Fig 7). However, one skin equivalent had large bulbous sebaceous glands that were innervated and opened to the skin surface. Myelinated nerve fibers were observed in the lower dermis of this graft; however, it was not determined whether they formed terminals on the sebaceous glands.

Graft Acceptance

Skin Equivalents: All cultured skin grafts were accepted at the time of bandage removal (10–20 d post-grafting). This assessment was based on graft size and gross appearance (Fig 1*B*), as well as on histology. At bandage removal, grafts were reduced to ~60% of their original size. The implants were clearly identifiable due to their opaqueness and protruding hairs. In contrast, the epidermis of the cultured skin was transparent and its surface had a "flaky" appearance. The dermis of the skin equivalent grafts maintained a homogeneous form of the gel (Fig 8). Furthermore, at the time of bandage removal, the largest open wound controls (0.625 cm^2) were not covered by epidermis; but wounds twice as large (1.26 cm^2) that were covered with cultured grafts possessed a thick and hyperkeratotic epidermis.

At various observational intervals (30–40 d and 80–90 d) eight animals (Table I; SE = 4, SE+ND = 2, SE+TD = 2) had grafts that underwent partial-rejection episodes. This was manifested as small areas of crusting and subsided after about a week, leaving the grafts reduced in size. Two animals had grafts (SE+ND = 1; SE+TD = 1) that eventually underwent total necrosis with scar formation. And three animals had minimal graft rejection with little or no crusting up to 83 d post-grafting (Table I; SE = 2, SE+TD = 1).

Autografts: Five animals had autografts that retained hair follicles and had minimal dermal reorganization. And four animals had grafts that became totally denuded and exhibited extensive dermal reorganization. All grafts experienced epidermal thickening.

Nerve Counts In an effort to evaluate whether re-innervation was enhanced in grafts with target implants, comparisons were made of the number of nerve profiles present in the dermis of cultured skin grafts and their distance from the epidermis. Counts and measurements were made in the dermis of the skin equivalents in regions between the implants. There were twice as many nerve profiles in the dermis of skin equivalents with touch-dome implants and 1.5 times more in the dermis of grafts with non-dome implants as there were in grafts comprised entirely of cultured skin. Furthermore, the nearest distance of nerves from the epidermal basement lamina in skin equivalents with touch-dome implants averaged 13 μm, 22 μm in skin equivalents with non-domal implants, and 26 μm in pure skin equivalents (n = 36 histologic sections from six animals, SE = 2, SE+ND = 2, SE+TD = 2; see Table I).

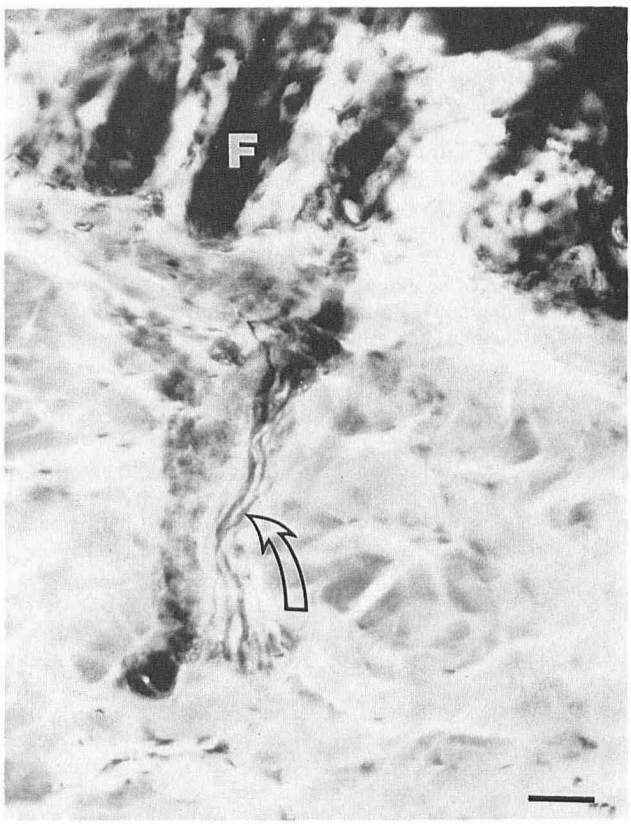

Figure 6. Implant region 83 d post-grafting. The *arrow* points to a silver-stained nerve bundle rising to hair follicles (F) in the touch-dome implant. The 2-mm implant was inserted into the skin equivalent at the time of grafting. *Scale bar,* 10 μm.

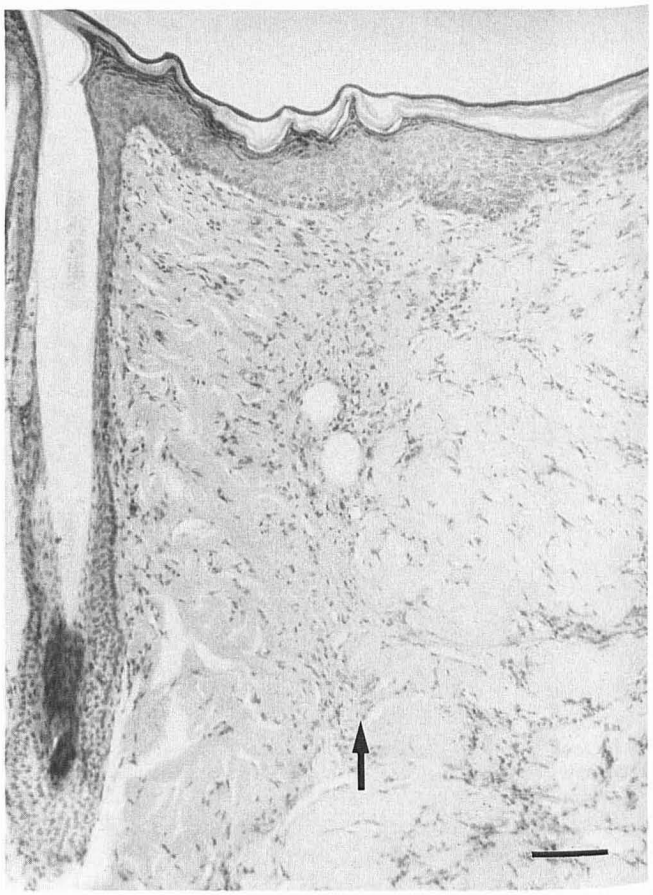

Figure 8. The junction *(arrow)* of a skin equivalent and normal skin 15 d post-grafting. The structure of the fibroblast contracted gel (to the right of the *arrow*) is readily distinguishable from the adjacent dermis of normal skin (left of the *arrow*). *Scale bar,* 100 μm.

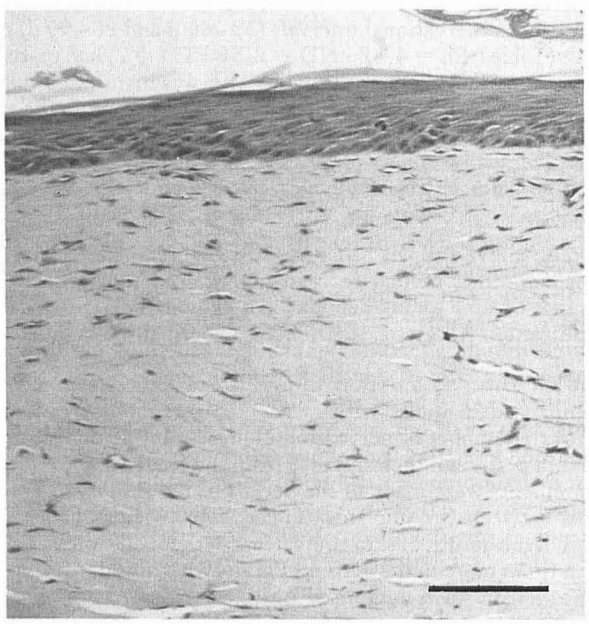

Figure 7. A skin equivalent stained with hematoxylin and eosin 1 year post-grafting. The epidermis is still hypertrophic and lacks adnexal structures. The dermis is composed of fine collagen fibrils. Fibroblasts and blood vessels are plethoric. *Scale bar,* 100 μm.

Nerve Recording To generate action potentials in nerves that innervated grafts comprised entirely of skin equivalent tissue, strong to moderate mechanical stimulation of the graft surface was required even in long-term animals (grafted more than 1 year). Lightly brushing the graft surface usually failed to elicit neural activity; however, rubbing it with a smooth glass rod, gently pinching, or stiffly brushing it produced a weak response. In one animal, two punctate "hot spots" were present on the graft surface. When pressure was applied to these spots with a glass rod or 80-mg von Frey hair, tonic activity was generated in the nerve. However, the nerve was silent when the remainder of the graft was rubbed with the rod or indented with a 1.0-g von Frey hair. Histology revealed that large bulbous sebaceous glands were present in the "hot spots" and that some were innervated by axon bundles.

Four animals with skin equivalent grafts with implants had greater functional innervation in the implants than in surrounding cultured skin (Table I; SE+ND = 2, SE+TD = 2). Figure 9 illustrates the sensory activation recorded from nerve fasicles in one rat that innervated normal skin (Fig 9, *1A,2A,3A*), an implanted biopsy without touch domes (Fig 9, *1B,2B,3B*), and skin equivalent areas (Fig 9, *1C,2C,3C*). Cutaneous fields were brushed (Fig 9, *1A–1C*), rubbed (Fig 9, *2A–2C*), or pinched (Fig 9, *3A–3C*). Light brushing of the implant produced an abundant discharge in the afferent nerves (Fig 9, *1B*) that was absent when surrounding cultured skin was brushed with equal intensity (Fig 9,*1C*). Only one of the two implanted biopsies remaining in this animal at 83 d post-grafting was re-innervated, and hair density of both implants was less than in adjacent normal skin.

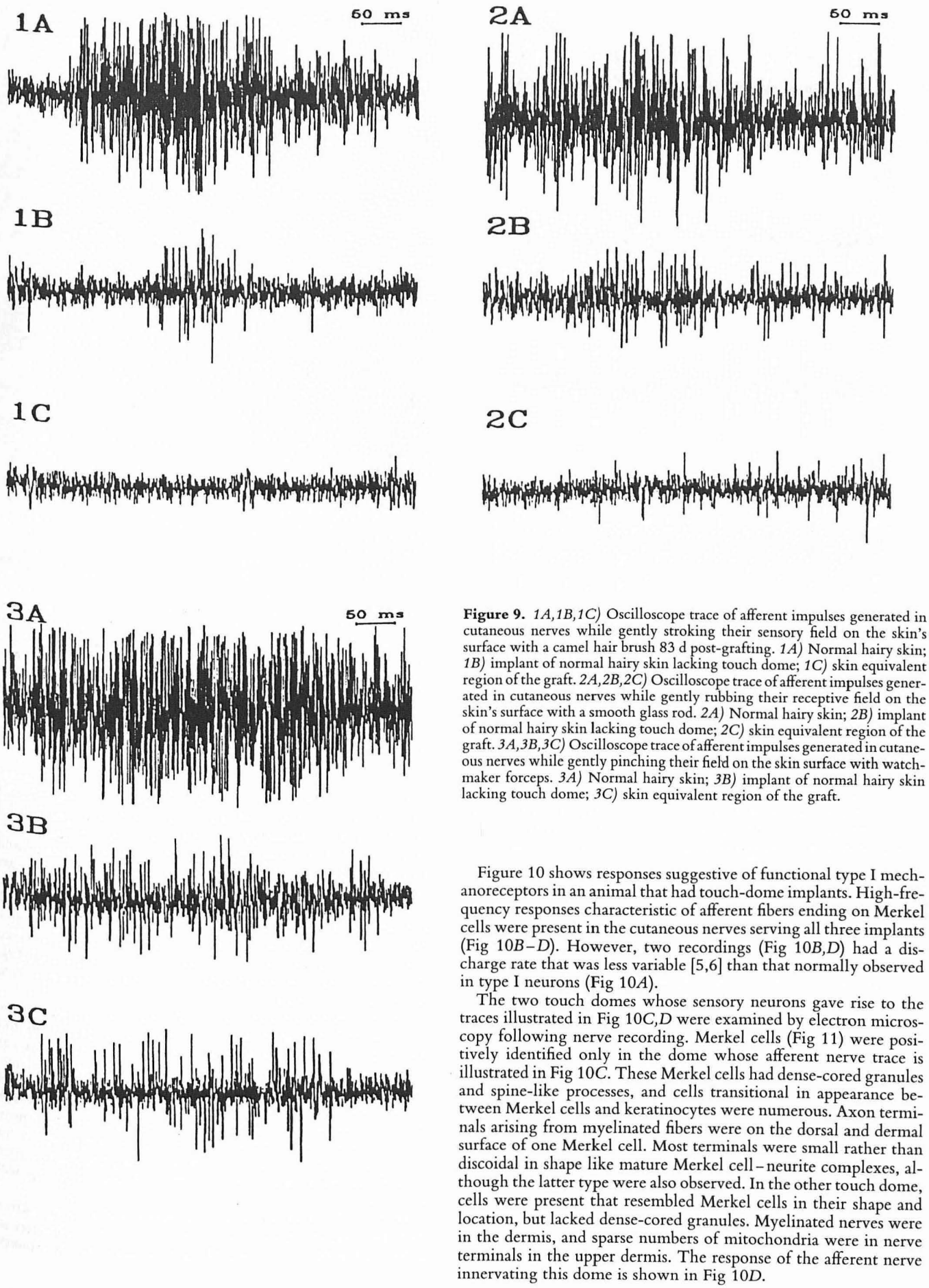

Figure 9. *1A,1B,1C)* Oscilloscope trace of afferent impulses generated in cutaneous nerves while gently stroking their sensory field on the skin's surface with a camel hair brush 83 d post-grafting. *1A)* Normal hairy skin; *1B)* implant of normal hairy skin lacking touch dome; *1C)* skin equivalent region of the graft. *2A,2B,2C)* Oscilloscope trace of afferent impulses generated in cutaneous nerves while gently rubbing their receptive field on the skin's surface with a smooth glass rod. *2A)* Normal hairy skin; *2B)* implant of normal hairy skin lacking touch dome; *2C)* skin equivalent region of the graft. *3A,3B,3C)* Oscilloscope trace of afferent impulses generated in cutaneous nerves while gently pinching their field on the skin surface with watchmaker forceps. *3A)* Normal hairy skin; *3B)* implant of normal hairy skin lacking touch dome; *3C)* skin equivalent region of the graft.

Figure 10 shows responses suggestive of functional type I mechanoreceptors in an animal that had touch-dome implants. High-frequency responses characteristic of afferent fibers ending on Merkel cells were present in the cutaneous nerves serving all three implants (Fig 10*B–D*). However, two recordings (Fig 10*B,D*) had a discharge rate that was less variable [5,6] than that normally observed in type I neurons (Fig 10*A*).

The two touch domes whose sensory neurons gave rise to the traces illustrated in Fig 10*C,D* were examined by electron microscopy following nerve recording. Merkel cells (Fig 11) were positively identified only in the dome whose afferent nerve trace is illustrated in Fig 10*C*. These Merkel cells had dense-cored granules and spine-like processes, and cells transitional in appearance between Merkel cells and keratinocytes were numerous. Axon terminals arising from myelinated fibers were on the dorsal and dermal surface of one Merkel cell. Most terminals were small rather than discoidal in shape like mature Merkel cell–neurite complexes, although the latter type were also observed. In the other touch dome, cells were present that resembled Merkel cells in their shape and location, but lacked dense-cored granules. Myelinated nerves were in the dermis, and sparse numbers of mitochondria were in nerve terminals in the upper dermis. The response of the afferent nerve innervating this dome is shown in Fig 10*D*.

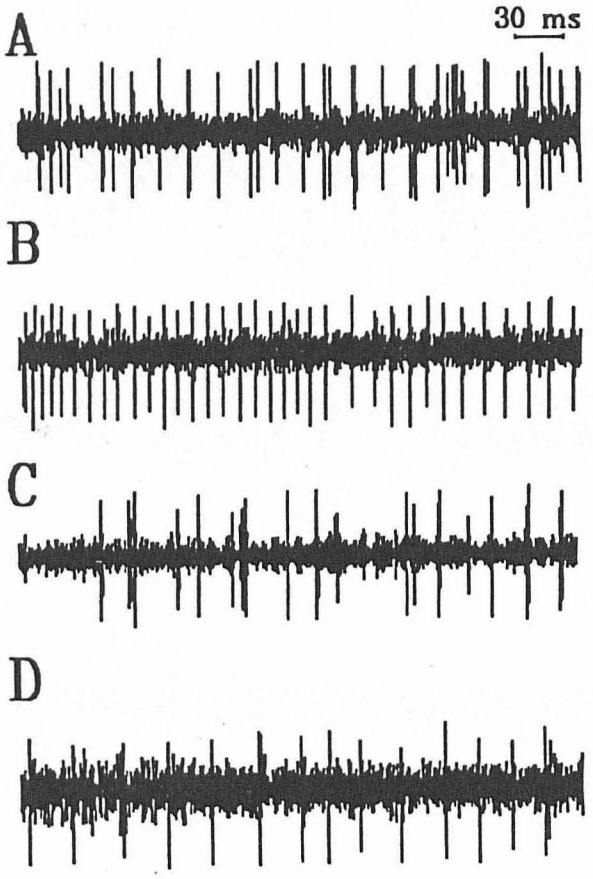

Figure 10. Type I responses generated in afferent nerves. *A)* Normal hairy skin. Responses of the afferent nerve innervating a touch dome stimulated with a hand-held glass rod. *B)* Type I responses obtained from thoracic nerve T_{10} serving the most rostral 2-mm punch biopsy implant. *C)* Type I responses recorded from thoracic nerve T_{11} serving the middle implant (see Fig 11). *D)* Type I responses recorded from thoracic nerve T_{12} serving the most caudal implant.

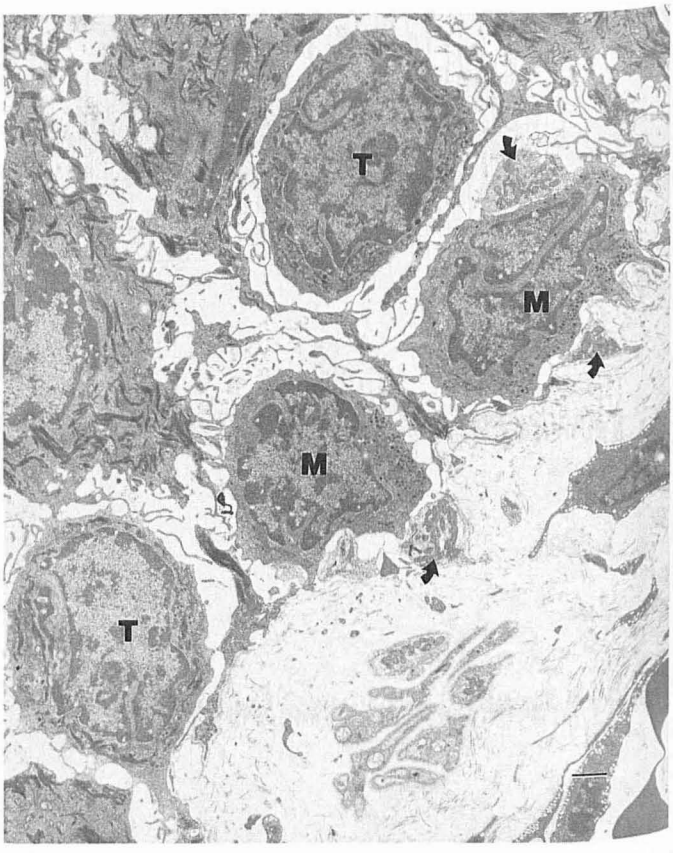

Figure 11. Electron micrograph of a touch dome from a 2-mm implant at 83 d post-grafting. Merkel cells (M) and transitional cells (T) are in the epidermis of the touch dome (see Fig 10*C* for the oscilloscope trace of the nerve recording of this dome). Nerve terminals *(arrows)* near individual Merkel cells are small and are packed with mitochondria. The terminal on the epidermal surface of the Merkel cell contains clear and dense cored vesicles as well as mitochondria. *Scale bar,* 1.0 μm.

One animal (Table I; SE+TD = 1) had denuded implants. There was no obvious increase in the amount of functional reinnervation in these implants as compared to surrounding cultured skin.

In Vitro Controls for Barriers to Nerve Growth At 6 d in culture, neurons from the dorsal root ganglia extended processes up to the epidermis. Groups of axons shared a common Schwann investment, and some were in direct contact with the gel. The axons' appearance in culture strongly resembled that of cutaneous neurons during in vivo development (Fig 12), in that individual axons were small and not yet enveloped by Schwann cells. Microtubules were well defined in the axoplasm, and the morphology of individual ganglion cell bodies was robust.

DISCUSSION

The results of the present investigation can be summarized as follows. 1) Grafts of cultured skin became innervated. 2) Innervation was enhanced if target tissue was present. 3) Activation similar to type I afferent responses was only observed in skin originally possessing touch domes; hence, this characteristic response was likely dependent upon factors unique to domal tissue besides the nerve alone. 4) The quality of the engraftment was vital to the return of sensory function.

Cultured skin grafts were functionally innervated. At the first observational point of 83 d, nerve fibers were numerous in the dermis and processes extended into the epidermis. However, light mechanical stimulation of skin-equivalent areas produced little neural response. Strong brushing, firm rubbing with a polished glass rod, or pinching was required to activate sensory neurons. Although measuring the time course of innervation was not an objective of this study, it is known that nerve terminals are found as early as 42 d at the epidermal basement lamina of cultured grafts derived from human skin on nude mice, and myelinated nerves are present in the deeper dermis [17]. Also, nerves in free-skin autografts made to rats reach the lower dermis by 28 d and hair follicles by 56 d [18]. It is therefore likely that innervation in this study occurred before the 83-d observation point.

The fact that nerves innervating 2-mm punch biopsies of normal skin inserted into cultured skin equivalents had sensitivity to light mechanical stimulation whereas those innervating the skin equivalent areas of the grafts did not, indicates that grafted implants provided a better environment for nerve recovery. The structure of end organs undoubtedly influenced the function of those nerves terminating on them, and trophic factors may have also been provided that caused regenerating fibers to mature [19,20]. Old Schwann tubes that remained in the implants following Wallerian degeneration of the nerve might have guided and nourished regenerating axons [21].

On the other hand, cultured skin usually lacks adnexal structures and hence any tropic or trophic factors they release that may attract regenerating fibers or cause them to mature. Nerve counts and

Figure 12. In vitro control. Dorsal root ganglia excised from newborn rat pups grew well and extended neuronal processes (*) throughout the collagen gels. Schwann cells (S) enveloped groups of axons at 6 days in vitro. *Scale bar,* 0.5 μm.

distance measurements suggested there was a trend toward increased overall innervation in grafts with implants, but because of the large animal-to-animal variability more studies employing greater numbers of animals are required. The level of re-innervation of skin autografts has also been reported to differ significantly between individual animals [22].

The finding of sebaceous glands in one of the first cultured skin equivalent grafts made was not necessarily surprising. The presence of fibroblasts among the keratinocytes suggests that the enzymatic epidermal separation technique was not as refined in early experiments. Sebaceous glands have a powerful ability to regenerate [23], and stem cells could easily have been included with the dissociated epidermis. Also, adnexal structures can regenerate if there is a proper ratio between fibroblasts and keratinocytes [24]. Because this graft had little or no crusting and was scored as having an excellent take, it is doubtful that the sebaceous glands were formed from remnant scar tissue. Interestingly, areas containing sebaceous glands were correlated with points on the skin surface where mechanical depression elicited tonic responses in the afferent nerves, and indicates the glands may have been targets for regenerating nerves.

Because responses characteristic of type I mechanoreceptors were only recorded from nerves re-innervating implants that originally possessed touch domes, the present study suggests that either i) the domal structure, or ii) Merkel cells, were necessary for type I activation. Therefore, our findings do not support the notion that type I nerve endings are the sole element required for the transduction of mechanical stimulation into type I neuronal discharge [25–28]. Rubbing implants without touch domes or graft areas comprised entirely of cultured skin with a glass rod did not elicit type I responses in any of the cutaneous afferent nerves even in long-term animals (> 1 year old; Table I). It is unlikely that type I neurons were selectively damaged by the grafting process, because in theory all of the cutaneous sensory nerve populations should have been present post-grafting, because only their terminal portions were excised when full-thickness graft sites were prepared. And, it is improbable that collagen gels were barriers for nerve regeneration, because nerve fibers grew through them in vitro. An alternative interpretation is that type I neurons require trophic factors provided by Merkel cells or touch domes in order to survive or function. In this case type I fibers could still be transducers; however, this capability would be dependent upon prior trophic induction (see also [29]). The actual role the Merkel cell plays in the mechanotransduction process is controversial; however, several investigations indicate it may be a transducer element [6,30–32].

Merkel cells and "transitional" cells [33–36] were found in only one of the two touch-dome implants examined by electron microscopy at 83 d post-grafting. And, most of the Merkel cell–neurite complexes were immature. If Merkel cells were present in the second touch dome they were likely few in number or altered in their appearance, because serial semithin sections were examined prior to selecting areas for ultrathin sectioning. However, a remnant dome-like structure was present in the second dome, and cells were observed that resembled Merkel cells in shape and location but lacked cytoplasmic dense-cored granules. These results suggest that although some component in the touch-dome structure was required for return of type I activation, Merkel cell–neurite complexes did not have to be fully mature and may not have been present. Alternatively, it is possible that only a small number of Merkel cell–neurite complexes needed to be present to activate the type I neurons, and hence might have been overlooked in the histologic examination. It is known that stimulation of a small complement of Merkel cell–neurite complexes in touch domes still results in type I responses in the nerve [6,37], but just how many complexes are required is speculative.

Many factors are involved in cutaneous recovery following grafting. Obviously, autografts contain all of their "target tissue components," yet autograft reinnervation is seldom if ever normal [38]. Autografts are often only partially re-innervated at their margins or patchily on their surface [20,23]. Tissue damage, bacterial invasion, pronounced scar formation, and decreased blood supply are all likely causes of poor graft take [39,40] with resulting impairment of neural function[41,42]. In the present investigation, cultured grafts that experienced little or no crusting subsequently had the best innervation. The shrinkage observed was comparable to that reported by other investigators [43] grafting cultured skin onto loose skinned animals whose skin heals primarily by wound contraction [44]. Because the grafts were autologous, it was impossible to determine whether graft epidermis was eventually replaced by adjoining keratinocytes. The graft sites were too large to have been covered by epidermis from surrounding skin at the time of bandage removal. The minor rejection episodes observed in this study may have been caused by delayed immune reactions to fetal bovine serum that was included in the growth medium [45,46]. The use of serum-free medium [47] to culture skin equivalents was reported to give a higher percentage of successful takes of human skin grafted to nude mice [17]. At present, many clinical trials employing cultured skin use serum fortified medium and a 3T3 fibroblast feeder layer [4,48]. If supportive studies come forth, a change to serum-free culture would be indicated not only for enhanced graft survival, but probably improved sensory function as well.

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